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Histone chaperone activity of Fanconi anemia proteins, FANCD2 and FANCI, is required for DNA crosslink repair

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Running title: Nucleosome dynamics by FANCD2-FANCI

Abstract

Fanconi anemia (FA) is a rare hereditary disorder characterized by genomic instability and cancer susceptibility. A key FA protein, FANCD2, is targeted to chromatin with its partner, FANCI, and plays a critical role in DNA crosslink repair. However, the molecular function of chromatin-bound FANCD2-FANCI is still poorly understood. In the present study, we found that FANCD2 possesses nucleosome-assembly activity *in vitro*. The mobility of histone H3 was reduced in FANCD2-knockdown cells following treatment with an interstrand DNA crosslinker, mitomycin C. Furthermore, cells harboring FANCD2 mutations that were defective in nucleosome assembly displayed impaired survival upon cisplatin treatment. Although FANCI by itself lacked nucleosome assembly activity, it significantly stimulated FANCD2-mediated nucleosome assembly. These observations suggest that FANCD2-FANCI may regulate chromatin dynamics during DNA repair.

Keywords: DNA repair/ FANCD2/ FANCI / Fanconi anemia/ histone chaperone

Introduction

Fanconi anemia (FA) is a rare hereditary disorder characterized by skeletal abnormalities, progressive bone marrow failure, and genomic instability accompanied by cancer susceptibility (Venkitaraman, 2004; Niedernhofer *et al*, 2005; Taniguchi and D'Andrea, 2006; Wang, 2007). FA mutant cells are highly sensitive to interstrand DNA crosslinking reagents, which induce stalled replication forks, suggesting that FA proteins promote the stabilization and restarting of the replisome (Thompson *et al*, 2005; Wang, 2007).

Thirteen genes, *FANCA*, *-B*, *-C*, *-D1 (BRCA2)*, *-D2*, *-E*, *-F*, *-G*, *-I*, *-J (BRIP1)*, *-L*, *-M*, and *-N (PALB2)*, corresponding to individual FA complementation groups, have been cloned (Thompson *et al*, 2005; Wang, 2007; Kee and D'Andrea, 2010; Garner and Smogorzewska, 2011; Kitao and Takata, 2011). Additionally, homozygous Rad51C mutations have recently been identified in a family with an FA-like disorder, as the *FANCO* gene (Vaz *et al*, 2010), and *SLX4* has been confirmed as the *FANCP* gene (Crossan *et al*, 2011; Kim *et al*, 2011; Stoepker *et al*, 2011). These FA gene products constitute a common DNA damage response pathway that is often referred to as the “FA pathway”. In this pathway, eight proteins, *FANCA*, *-B*, *-C*, *-E*, *-F*, *-G*, *-L*, and *-M*, and three *FANCA*-associated polypeptides (FAAPs) form the FA core E3 ligase complex (Garcia-Higuera *et al*, 2001; Wang, 2007; Ali *et al*, 2012; Kim *et al*, 2012; Leung *et al*, 2012). On the other hand, *FANCD2* and *FANCI* associate with each other to form a different complex, called the ID complex (Sims *et al*, 2007; Smogorzewska *et al*, 2007).

Upon DNA damage during S phase, multiple phosphorylations of *FANCI* trigger the monoubiquitination of *FANCD2* and *FANCI* by the FA core complex (Ishiai *et al*, 2008). The monoubiquitinated ID complex is then targeted to the chromatin, where it plays a critical role in DNA repair pathways, such as homologous recombination and

translesion synthesis (Matsushita *et al*, 2005; Thompson *et al*, 2005; Yamamoto *et al*, 2005; Wang, 2007; Kee and D'Andrea, 2010; Garner and Smogorzewska, 2011; Kitao and Takata, 2011). Recent studies indicated that monoubiquitinated FANCD2 (and FANCI) recruit the FAN1 nuclease, which possesses endo- and exonuclease activities, providing a partial explanation for their roles in DNA repair (Kratz *et al*, 2010; MacKay *et al*, 2010; Smogorzewska *et al*, 2010; Yoshikiyo *et al*, 2010). Monoubiquitinated FANCD2 also reportedly recruits SLX4 (Garner and Smogorzewska, 2011; Yamamoto *et al*, 2011), which is considered to function as a scaffold that interacts with the other nucleases, SLX1, XPF, and MUS81 (Fekairi *et al*, 2009; Svendsen *et al*, 2009; Yamamoto *et al*, 2011). Furthermore, a recent report found that FANCD2 itself might have exonuclease activity (Pace *et al*, 2010). However, whether chromatin-bound FANCD2 and FANCI have any additional functions remains to be determined.

The nucleosome is the fundamental repeating unit of chromatin (Wolffe, 1998). Four core histones, H2A, H2B, H3, and H4, are the protein components of the nucleosome. H2A forms a specific dimer with H2B (H2A/H2B dimer), and H3 forms a specific dimer with H4 (H3/H4 dimer). During nucleosome assembly, two H3/H4 dimers (H3/H4 tetramer) are first deposited on DNA, forming a tetrasome, in which the DNA is wrapped around the H3/H4 tetramer. Two H2A/H2B dimers are then incorporated into the tetrasome to form the mature nucleosome, in which about 150 base pairs of DNA are wrapped around a histone octamer, containing two each of the H2A/H2B and H3/H4 dimers. In cells, nucleosomes are dynamically assembled and disassembled during the replication, transcription, recombination, and repair processes, and such nucleosome dynamics are accomplished with the aid of histone chaperones and/or ATP-dependent chromatin remodeling factors (Avvakumov *et al*, 2011).

In the present study, we purified the human and chicken FANCD2 proteins, and found that FANCD2 possesses nucleosome-assembly activity *in vitro*. We also purified FANCI, and showed that it significantly stimulated FANCD2-mediated nucleosome assembly, although FANCI itself lacked nucleosome assembly activity. A histone-binding domain was mapped in the chicken FANCD2 C-terminal region (residues 1268-1439). The FANCD2 mutants, in which either the histone-binding domain was deleted or the Arg1336 and Lys1346 residues were replaced by Ala, were significantly defective in nucleosome assembly *in vitro*, and cells bearing these mutants displayed impaired survival upon cisplatin treatment *in vivo*. Furthermore, a disease-related mutation, human FANCD2(R302W) (Timmers *et al*, 2001), compromised histone dynamics, and the corresponding chicken FANCD2(R305W) also showed impaired histone chaperone activity. These data suggest that the histone chaperone activity of FANCD2 is crucial for the histone dynamics and the DNA crosslink repair in cells.

Results

Human FANCD2 promotes nucleosome assembly

In a proteome analysis to search for proteins in HeLa cell extracts that bind to the histone H3/H4 complex (Supplementary Figure S1), we unexpectedly detected human FANCD2 (hFANCD2) as a candidate interacting protein. Indeed, hFANCD2 was efficiently captured from a HeLa cell extract, using H3/H4 beads (Figure 1A). We purified hFANCD2 as a recombinant protein expressed in insect cells (Supplementary Figure S2A), and confirmed that purified hFANCD2 also bound to H3/H4 (Figure 1B). The

hFANCD2-H3/H4 binding was also detected in the presence of DNaseI (Figure 1B, lane 5), indicating that the interaction is not mediated by DNA contamination. These results indicated that hFANCD2 directly binds to H3/H4, which prompted us to examine its nucleosome assembly activity.

We tested hFANCD2-mediated nucleosome assembly by a topological assay, using relaxed circular DNA in the presence of topoisomerase (Figure 1C). The extent of nucleosome formation was assessed by analyzing the superhelicity of circular DNA fractionated through an agarose gel, because negative supercoils are introduced when nucleosomes are formed. As shown in Figure 1C, the number of superhelical turns in the DNA substrate increased with greater amounts of hFANCD2. The faster migration of the DNA substrate was not due to DNA degradation (Supplementary Figure S2B). Therefore, hFANCD2 actually promoted nucleosome assembly *in vitro*. We next performed the nucleosome assembly assay with a short DNA fragment, to directly detect the nucleosomes by an electrophoretic mobility shift assay. hFANCD2 stimulated the nucleosome assembly in this assay (Figure 1D, lanes 6-9). The nucleosome assembly activity of hFANCD2 was slightly lower than that of human Nap1, which is a prominent nucleosome assembly protein (Figure 1D). These biochemical results suggest that FANCD2 may regulate chromatin reorganization during DNA repair in higher eukaryotes.

Chromatin-bound FANCD2 is known to be monoubiquitinated, and the ubiquitin moiety may function to recruit its associated nucleases (Fekairi *et al*, 2009; Svendsen *et al*, 2009; Kratz *et al*, 2010; MacKay *et al*, 2010; Smogorzewska *et al*, 2010;

Yoshikiyo *et al*, 2010; Yamamoto *et al*, 2011). Therefore, we tested whether FANCD2 monoubiquitination affects the nucleosome assembly activity. For this purpose, we utilized the chicken FANCD2 protein (cFANCD2) (Yamamoto *et al*, 2005), which was bacterially expressed and purified to homogeneity (Supplementary Figure S2C). We then prepared monoubiquitinated cFANCD2, using purified components for the conjugation (i.e., FANCL, UBE2T, E1, and ubiquitin; Supplementary Figure S2D-F). As we previously reported, the cFANCD2 monoubiquitination was robustly enhanced in the presence of DNA (Sato *et al*, 2012), and about 40% of cFANCD2 was monoubiquitinated in this study (Supplementary Figure S2F). This monoubiquitinated cFANCD2 fraction was purified, and was subjected to the topological assay. However, we did not find a clear difference in the nucleosome assembly activities between the fractions containing monoubiquitinated cFANCD2 and the monoubiquitination-deficient cFANCD2(K563R) mutant (Supplementary Figure S2G). Therefore, the monoubiquitination does not affect the activity. However, this could be due to the incomplete monoubiquitination of cFANCD2. Therefore, we prepared the monoubiquitination-mimicking version of FANCD2, by genetically fusing FANCD2(K563R) with ubiquitin to create FANCD2(K563R)-Ub (Supplementary Figure S2H), which is known to complement the DNA-repair defective phenotype in the *FANCD2*^{-/-} DT40 cells (Matsushita *et al*, 2005). We found that purified FANCD2(K563R)-Ub possessed similar histone-binding and nucleosome-assembly activities to those of cFANCD2 (Supplementary Figure S2I and J). These results suggested that the FANCD2 monoubiquitination may not be directly involved in the nucleosome assembly.

The C-terminal region of FANCD2 is responsible for interacting with histone H3/H4 and for promoting nucleosome assembly

To gain further insights into the molecular mechanism and the functional relevance of the nucleosome assembly activity of FANCD2, we first searched for the FANCD2 region that interacts with the H3/H4 complex. We subjected cFANCD2 to limited proteolysis, and two fragments, cFANCD2(1-1167) and cFANCD2(1-1389), were identified (Figure 2A). These fragments lacked the acidic region, which is composed of the C-terminal 50 amino acid residues of FANCD2. In addition, FANCD2(1268X), which also lacks the C-terminal region, is present in FA patients (Fanconi Anemia Mutation Database, <http://www.rockefeller.edu/fanconi/mutate/>), suggesting the functional importance of the FANCD2 C-terminal region. As histone chaperones are generally acidic, we investigated whether this acidic C-terminal region is essential for histone binding. The C-terminal deletion mutants, cFANCD2(1-1167), cFANCD2(1-1267), and cFANCD2(1-1389), were expressed as GFP-tagged forms in HEK293T cells, and their H3/H4 binding activity was examined by a pull-down assay, using the H3/H4 beads. We found that cFANCD2(1-1167) and cFANCD2(1-1267) displayed diminished H3/H4 binding (Figure 2B, lanes 3 and 4). In contrast, cFANCD2(1-1389) retained residual H3/H4 binding activity (Figure 2B, lane 2). Consistently, cFANCD2(1-1167) and cFANCD2(1-1267) showed significant defects in nucleosome assembly (Figure 2C, lanes 13-17 and 18-22, and D; Supplementary Figure S3A). cFANCD2(1-1389) was completely proficient in the nucleosome assembly activity under low protein concentration conditions (Figure 2C,

lanes 8-12, and D; Supplementary Figure S3B). These observations suggest that the C-terminal region of FANCD2 (amino acids 1268-1389) is important for interacting with H3/H4 and for promoting nucleosome assembly. It should be noted that cFANCD2(1-1389) was defective in nucleosome assembly under high protein concentration conditions (Figure 2C, lanes 10-11, and D; Supplementary Figure S3C). This may reflect the biochemical property of cFANCD2(1-1389), which tends to form large protein-DNA aggregates that are unable to enter an agarose gel during electrophoresis (Supplementary Figure S3D).

Since the cFANCD2 C-terminal deletion may induce the improper folding of the cFANCD2 structure, we next performed the H3/H4 binding and nucleosome assembly experiments with cFANCD2 point mutants. Based on the amino acid conservation among the human, mouse, chicken, frog, fish, and fly FANCD2 proteins, we mutated the conserved amino acid residues, which might be functionally important (Figure 2A). We found that the FANCD2(R1336A/K1346A) mutant, in which the Arg1336 and Lys1346 residues are replaced by Ala, was significantly defective in nucleosome assembly *in vitro*, while another mutant, cFANCD2(D1350A/E1365A/E1382A), in which the Asp1350, Glu1365, and Glu1382 residues are replaced by Ala, did not affect the nucleosome assembly activity (Figure 2A, E, and F; Supplementary Figure S3E). Consistently, the histone-binding activity was substantially reduced in cFANCD2(R1336A/K1346A) (Figure 2B lane 11), but not in cFANCD2(D1350A/E1365A/E1382A) (Figure 2B, lane 10). These results strongly support the conclusion that FANCD2 promotes nucleosome assembly through its

C-terminal histone-binding domain.

Finally, we tested the histone binding of the C-terminal cFANCD2 fragments, cFANCD2(669-1439) and cFANCD2(953-1439), which contain the C-terminal amino acid residues 669-1439 and 953-1439, respectively (Figure 2A). These cFANCD2 fragments were identified by a protease mapping experiment. As expected, the cFANCD2(669-1439) and cFANCD2(953-1439) fragments both efficiently bound to histones (Figure 2B, lanes 5 and 6). Surprisingly, cFANCD2(953-1439), which contained only one-third of cFANCD2, promoted nucleosome assembly (Figure 2C, lanes 23-27, and D). Therefore, we concluded that the histone-binding domain is located in the C-terminal region of FANCD2.

FANCD2 mediates histone mobilization in living cells in a DNA damage-dependent manner

To determine whether FANCD2 plays a role in histone dynamics in living cells during DNA repair, we knocked down hFANCD2 in HeLa cells expressing histone H3-GFP (Kimura and Cook, 2001), using small inhibitory RNA (siRNA). Three days after the transfection of the specific siRNA, the level of hFANCD2 had decreased substantially, to <10% of the normal level (Supplementary Figure S4A and B). Using these cells, the mobility of H3 was analyzed by fluorescence recovery after photobleaching (FRAP) (Kimura *et al*, 2006). The recovery kinetics (the curve shapes) of the exchanging fractions were similar in the hFANCD2-knockdown and control cells (Figure 3A), suggesting that hFANCD2 does not play a major role in H3 assembly or exchange under

normal conditions. As FA mutant cells display significant sensitivity to interstrand DNA crosslinking reagents, such as mitomycin C (MMC) (Niedernhofer *et al*, 2005; Thompson *et al*, 2005; Wang, 2007; Kee and D'Andrea, 2010; Garner and Smogorzewska, 2011; Kitao and Takata, 2011), we next tested the effect of MMC on the H3 mobility in the hFANCD2 knockdown HeLa cells. Interestingly, the recovery of H3-GFP in the hFANCD2 knockdown cells was clearly slower in the presence of MMC (Figure 3B). Similar results were obtained with a different FANCD2-specific siRNA (Supplementary Figure S4C). The slower H3-GFP exchange observed in the MMC-treated FANCD2-knockdown cells could be due to a different cell cycle distribution, since the FA deficient cells may be arrested at S and/or G2 due to the deficiency of DNA crosslink repair. We therefore performed FRAP experiments in cells stably expressing both H3-GFP and mCherry-tagged PCNA, which shows characteristic patterns representing replication and repair foci (Leonhardt *et al*, 2000). We first examined the mobility of H3-GFP in different cell cycle stages under the normal growth conditions. The H3-GFP mobility in PCNA foci-positive (S-phase) cells did not differ from that in PCNA foci-negative cells (Figure 3C; Supplementary Figure S4D). In the presence of MMC, the PCNA foci-positive cells were indeed enriched by the hFANCD2 knockdown, but the mobility of H3-GFP was still slower than that in the MMC-treated PCNA foci-positive control cells (Figure 3D; Supplementary Figure S4D). Therefore, the reduced histone H3 mobility in the MMC-treated hFANCD2 knockdown cells does not appear to be attributable to a difference in the cell cycle phase. Furthermore, the slower H3-GFP recovery in the presence of MMC was also detected in the hFANCA knockdown cells, in

which the damage-dependent focus formation of hFANCD2 on chromosomes was significantly inhibited (Supplementary Figure S4E-H). These data suggest that hFANCD2 may mediate nucleosome assembly and/or histone exchange in human cells, in a damage-dependent manner.

The C terminal histone-binding region of FANCD2 is important for the DNA repair mediated by the FA pathway

To determine whether the histone assembly activity levels of the FANCD2 mutants *in vitro* correlate with their DNA repair activities *in vivo*, we expressed them in *FANCD2*^{-/-} DT40 cells, and exposed the cells to cisplatin in a colony survival assay. As shown in Figure 4A, full length cFANCD2, cFANCD2(1-1389), and cFANCD2(D1350A/E1365A/E1382A) rescued the cisplatin-sensitive phenotype of the *cFANCD2*^{-/-} cells, in contrast to cFANCD2(1-1167), cFANCD2(1-1267), and cFANCD2(R1336A/K1346A). To ensure the chromatin targeting of these cFANCD2 mutants, we repeated this assay with the cFANCD2 mutants expressed as fusions with histone H2B, since cFANCD2(1-1167), cFANCD2(1-1267), and cFANCD2(R1336A/K1346A) were not monoubiquitinated (Figure 4B), probably due to the weakened interaction with FANCL, which was identified as the catalytic E3 subunit for FANCD2 monoubiquitination (Meetei et al, 2003) (Supplementary Figure S5A). We confirmed that the fusion of H2B to cFANCD2 and cFANCD2(1-1267) did not affect their nucleosome assembly activities (Supplementary Figure S5B-E). Although substantial amounts of cFANCD2(1-1167)-H2B, cFANCD2(1-1267)-H2B, and

cFANCD2(R1336A/K1346A)-H2B were detected at the chromatin (Figure 4D), the mutants still could not complement the cisplatin sensitivity of *cFANCD2*^{-/-} cells, in contrast to the H2B fusion proteins with the full-length cFANCD2 and cFANCD2(K563R), bearing a point mutation at the monoubiquitination site (K563R) (Figure 4C) (Matsushita *et al*, 2005). These results strongly suggest that the nucleosome assembly activity, which depends on the C-terminal region of FANCD2, might be crucial for the DNA repair mediated by the FA pathway. Notably, these cFANCD2 mutants were able to interact with the cFANCI protein in chromatin, as detected by anti-GFP immunoprecipitation followed by western blotting (Figure 4D), and as further supported by the results of *in vitro* binding experiments (Supplementary Figure S7F-H).

A disease-related FA mutant, human FANCD2(R302W), and its chicken counterpart, cFANCD2(R305W), are monoubiquitinated but do not facilitate histone exchange

As shown in Figure 4B, the C-terminally deleted or point FANCD2 mutants, which were defective in nucleosome assembly, were also defective in monoubiquitination, probably due to their weakened interactions with FANCL (Supplementary Fig. S5A). This suggested that the histone-binding and FANCL-binding regions may partially overlap. To provide evidence that the FANCD2 histone-chaperone activity functions in DNA repair independently of its monoubiquitination, a FANCD2 mutant, in which the histone-chaperone and monoubiquitination activities are separated, would be useful. We reasoned that such mutations might be found outside the putative histone-binding site. We found that the chicken FANCD2 R305W mutation (Arg305 to Trp), which

corresponds to a disease-related mutation (human FANCD2 R302W) in Fanconi anemia (Timmers *et al*, 2001), is such a separation mutation. cFANCD2(R305W) exhibited reduced histone binding (Figure 2B, lane 8), and was defective in the nucleosome assembly activity (Figure 5A and B). In contrast, cFANCD2(R305W) was proficient in its monoubiquitination (Figure 5C) and chromatin targeting activities (Supplementary Figure S6A). Interestingly, cFANCD2(R305W) was moderately defective in the repair of DNA damage induced by cisplatin (Figure 5D), although its binding to cFANCI was proficient *in vivo* (Supplementary Figure S6A) and *in vitro* (Supplementary Figure S7E). The DNA repair deficiency was also observed when H2B-fused cFANCD2(R305W) was expressed in the cFANCD2^{-/-} cells (Figure 5E). These results indicated that the DNA-repair defect observed in the cFANCD2^{-/-} cells expressing cFANCD2(R305W) may be accounted for by the defective histone chaperone activity of cFANCD2(R305W). Therefore, the histone chaperone activity of FANCD2 may be required in the steps after the FANCD2 monoubiquitination and chromatin targeting, during DNA repair by the FA pathway (Figure 8B).

To test whether the histone chaperone activity of FANCD2 functions in histone dynamics in living cells, we expressed mCherry-tagged hFANCD2, hFANCD2(R302W), or hFANCD2(K561R) in H3-GFP-expressing HeLa cells, in which the endogenous hFANCD2 was knocked down by siRNA (Supplementary Figure S6B). We compared the H3-GFP mobility between mCherry-positive and -negative cells. The mobility of H3-GFP was efficiently restored with the expression of wild-type hFANCD2 (Figure 6A), but not with the monoubiquitination-deficient hFANCD2(K561R) mutant (Figure 6B,

top panel), consistent with its chromatin targeting deficiency (diffusing within the nucleus; Figure 6B, bottom panels). Interestingly, hFANCD2(R302W) also failed to restore the H3-GFP mobility (Figure 6C, top panel), although its chromatin targeting was not defective (forming nuclear foci like the wild-type hFANCD2; Figure 6C, bottom panels). Together with the DNA-repair deficiency of the cFANCD2^{-/-} cells expressing cFANCD2(R305W), these results support the view that the histone-chaperone activity of FANCD2 is important in the DNA repair by the FA pathway.

FANCI stimulates nucleosome assembly by FANCD2

Finally, we examined the involvement of FANCI in nucleosome assembly, using purified chicken FANCI (cFANCI) (Figure S7A and B), which could form a stable complex with cFANCD2 (Figure 7A). Although FANCI shares significant homology with FANCD2 (Sims *et al*, 2007; Smogorzewska *et al*, 2007), purified cFANCI alone did not efficiently promote nucleosome assembly (Figure 7B). However, cFANCI clearly stimulated the cFANCD2-mediated nucleosome assembly at low cFANCD2 concentrations, at which cFANCD2 itself did not promote detectable levels of nucleosome assembly (Figure 7C and D). cFANCI did not form a complex with the major histone chaperone Nap1, and did not stimulate its nucleosome assembly (Supplementary Figures 7C and D), suggesting that it may specifically stimulate the cFANCD2-mediated nucleosome assembly. In addition, cFANCI stimulated the nucleosome assembly activity of cFANCD2(1-1389), which retained H3/H4 binding ability, but not that of cFANCD2(1-1267) (Supplementary Figure S7I). Therefore, the ID complex may facilitate nucleosome reorganization during

DNA repair.

Discussion

In the present study, we found that the purified FANCD2 protein promotes nucleosome assembly *in vitro*, and that its partner, FANCI, significantly stimulates this activity. We also discovered that the C-terminal region of FANCD2 is critical for the nucleosome assembly activity, and showed that the FANCD2 deletion and point mutants are defective in both nucleosome assembly *in vitro* and tolerance to cisplatin treatment *in vivo*. These findings provide novel insights into the function of the ID complex in chromatin.

The crystal structure of the mouse ID complex revealed that FANCD2 is composed of seven subdomains, solenoid 1 (S1), helical domain 1 (HD1), solenoid 2 (S2), helical domain 2 (HD2), solenoid 3 (S3), solenoid 4 (S4), and the C-terminal acidic region (Joo *et al.* 2011). In the present study, we mapped the histone-binding region within the S4 domain of FANCD2. The FANCD2 S4 domain is separate from the FANCI-binding surface, and is largely accessible (Joo *et al.*, 2011). In the S4 domain, the cFANCD2 Arg1336 and Lys1346 residues are perfectly conserved among the human, mouse, chicken, frog, fish, and fly FANCD2 proteins (Figure 2A), and are located on the concave surface (Figure 8A; Joo *et al.*, 2011). Our mutational analysis revealed that the cFANCD2 Arg1336 and Lys1346 residues are essential for nucleosome assembly *in vitro* and cisplatin resistance *in vivo*. In the crystal structure, the side chains of the mouse FANCD2 Arg1334 and Lys1344 residues, corresponding to the cFANCD2 Arg1336 and

Lys1346 residues, respectively, are completely exposed to the solvent on the concave surface (Figure 8A). Therefore, the concave surface of the FANCD2 S4 domain may be its histone-binding region.

In cFANCD2, Arg1336 and Lys1346 are positively charged residues, which are considered to be less important for the histone binding of the acidic histone chaperone, Asf1 (English *et al*, 2006; Natsume *et al*, 2007). However, the involvement of positively charged residues in histone binding has been reported in the histone chaperone HJURP, which is the specific chaperone for the histone H3 variant, CENP-A (Dunleavy *et al*, 2009; Foltz *et al*, 2009). In the complex of HJURP with histone H4 and CENP-A, polar interactions exist between the negatively charged Glu96 of CENP-A and the two positively charged Arg32 and Lys39 residues of HJURP, and also between Glu107 of CENP-A and Arg28 of HJURP (Hu *et al*, 2011). Interestingly, the Glu96 and Glu107 residues of CENP-A are both conserved in histone H3 (Tachiwana *et al*, 2011a). Therefore, the histone-binding mechanism of FANCD2 may be similar to that of HJURP.

In the present study, we found that cFANCD2(R305W) was clearly defective in reversing the cisplatin sensitivity of the FANCD2^{-/-} DT40 cells, but was proficient in its monoubiquitination *in vivo*. Interestingly, cFANCD2(R305W) was significantly defective in both the histone binding and nucleosome assembly activities. In addition, the corresponding human FANCD2(R302W) mutant could not rescue the decreased histone H3 mobility in the FANCD2-knockdown cells. These findings indicate that the histone-chaperone activity of FANCD2 is actually involved in the DNA crosslink repair by the FA pathway, and strongly suggest that FANCD2 regulates nucleosome dynamics

after its monoubiquitination. The C-terminal cFANCD2 mutants tested in this study showed more severe cisplatin sensitivity than cFANCD2(R305W), even when localized in the chromatin by the H2B fusion. The residual histone-chaperone activity of cFANCD2(R305W), which was barely detectable in the present nucleosome formation assay, may partially complement the cisplatin sensitivity of the cFANCD2^{-/-} cells. In the crystal structure of mouse FANCD2, the side chain of the corresponding Arg300 directly hydrogen bonds with Asp379 (Joo *et al*, 2011), suggesting that the Arg300-Asp379 interaction may be important in the tertiary structure of the protein. Therefore, the Arg to Trp substitution found in cFANCD2(R305W) or hFANCD2(R302W) may cause a large structural change, which may allosterically impair the histone binding activity of FANCD2.

FANCI-FANCD2 is reportedly required for nucleolytic incisions in a replicating plasmid carrying an interstrand crosslink in a frog egg extract (Knipscheer *et al*, 2009). One can envision that the ID complex-mediated chromatin remodeling may regulate the incision step by promoting the recruitment of nucleases (Figure 8B, I) (e.g. FAN1, Mus81, and XPF) (Nomura *et al*, 2007; Bhagwat *et al*, 2009; Fekairi *et al*, 2009; Svendsen *et al*, 2009; Hicks *et al*, 2010; Kratz *et al*, 2010; MacKay *et al*, 2010; Smogorzewska *et al*, 2010; Yoshikiyo *et al*, 2010; Yamamoto *et al*, 2011). In this step, the proper incision by the repair nucleases may occur on the single-stranded DNA region (Figure 8B, I, arrowhead), while the improper digestion of intact DNA strands may be restricted by the nucleosomes formed by the ID complex (Figure 8B, I, dashed line). Secondly, in the FA pathway, homologous recombination repair may be promoted after FANCD2 assembly

into chromatin. The nucleosome remodeling by the ID complex may facilitate the DNA recombination reaction mediated by RAD51, which is the key protein for homologous recombinational repair (Figure 8B, II). Finally, the histone assembly activity of the ID complex may simply be important for chromatin reformation, following crosslink removal and DNA repair (Figure 8B, III). In addition to the direct interaction of the ID complex with histones, presented here, several chromatin modifiers have been suggested to interact with FA proteins. For example, a histone acetyltransferase, TIP60, reportedly interacts with FANCD2 (Hejna *et al*, 2008). Thus, the function of FANCD2 may be tightly coupled with those of other chromatin remodeling factors in chromatin dynamics, to facilitate DNA crosslink repair.

Materials and Methods

Purifications of FANCD2, FANCI, FANCL, UBE2T, and Nap1

Experimental procedures are described in Supplementary Materials and Methods. The protein concentration was determined by the Bradford method (Bradford 1976), using bovine serum albumin as the standard protein.

Purification of recombinant human H3/H4 and H2A/H2B complexes

Human histones H2A, H2B, H3, and H4 were overexpressed in *Escherichia coli* cells as His₆-tagged proteins, as described (Tanaka *et al*, 2004; Tachiwana *et al*, 2008). The purification and preparation of the histone H3/H4 and H2A/H2B complexes were

performed as previously described (Tachiwana *et al*, 2010; 2011b). The His₆-tag was removed during the purification steps.

Proteome analysis

The purified recombinant human histone H3/H4 complex was covalently conjugated with Affi-Gel 10 beads (Bio-Rad), according to the manufacturer's protocol. Briefly, the histone H3/H4 beads were incubated with a chromatin extract from HeLa cells at 4°C. The beads were washed with 10 mM PIPES buffer (pH 7.0), containing 1 mM MgCl₂, 1 mM EDTA, 0.01% Triton X-100, 300 mM sucrose, and 0.1 M NaCl. The proteins bound to the beads were fractionated by SDS-PAGE. Each lane was cut into nine pieces, and was further treated with trypsin. To identify the peptide fragments, the samples were analyzed by liquid chromatography/tandem mass spectrometry, as previously described (Nozawa *et al*, 2010). The raw data files were analyzed by the Mascot software (Matrix Science).

Pull-down assay with histone-conjugated beads

The histone H3/H4 complex was covalently conjugated with Affi-Gel 10 beads (Bio-Rad), according to the manufacturer's protocol. The histone H3/H4 beads were incubated with a HeLa whole cell extract (3 mg of protein), and the beads were washed three times with 100 µL of washing buffer, containing 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSF and Protease Inhibitor Cocktail (Nacalai Tesque). The hFANCD2 that copelleted with the histone H3/H4 beads was fractionated by 8% SDS-PAGE, and was detected by western blotting with the

hFANCD2-specific mouse monoclonal antibody (FI17, Santa Cruz Biotechnology, Inc.).

For the recombinant hFANCD2-binding assay, the histone H3/H4 beads (15 μ L) were incubated with purified hFANCD2, with or without 10 U of DNaseI (TOYOBO), at 23°C for 120 min. The beads were then washed three times with 100 μ L of 10 mM PIPES-KOH buffer (pH 7.0), containing 0.5 M NaCl, 0.3 M sucrose, 1 mM $MgCl_2$, and 0.01% Triton X-100. The proteins bound to the beads were separated by 15% SDS-PAGE, and were visualized by Coomassie Brilliant Blue staining.

For the cFANCD2-binding assay, 293T cells were transiently transfected with GFP-cFANCD2 mutant plasmids, using Lipofectamine2000 (Invitrogen). Cells were disrupted in lysis buffer (Yamamoto *et al*, 2005), and after the lysate was incubated with the histone H3/H4 beads (7.5 μ L) at 4°C for 3 hr, the beads were washed four times with lysis buffer. The proteins were separated by 6% SDS-PAGE, and were detected by western blotting with an anti-GFP Ab (Clontech).

Topological assay for nucleosome formation

Experimental procedures are described in Supplementary Materials and Methods.

Gel shift assay for nucleosome formation

The H2A/H2B complex (8 ng/ μ L) and the H3/H4 complex (8 ng/ μ L) were pre-incubated with hFANCD2 or hNap1 at 23°C for 10 min. The nucleosome assembly reaction was initiated by the addition of the 195 bp DNA (8 ng/ μ L) containing the *Lytechinus variegates* 5S ribosomal RNA gene (Osakabe *et al*, 2010) in a 10 μ L reaction mixture,

containing 20 mM Tris-HCl (pH 8.0), 140 mM NaCl, 7% glycerol, and 1 mM dithiothreitol. The reaction was continued at 23°C for 60 min, followed by a further incubation at 42°C for 60 min to eliminate the non-specific DNA binding by free histones. The samples were then separated by 6% PAGE in 0.2x TBE buffer (18 mM Tris base, 18 mM boric acid, and 0.4 mM EDTA). DNA was visualized by ethidium bromide staining.

Pull-down assay with GST-cFANCL and cFANCD2

Purified cFANCD2 (6 µg) and GST-cFANCL (5 µg) were incubated at 30°C for 60 min in a 100 µL reaction mixture, containing 20 mM Tris-HCl (pH 8.0), 10% glycerol, 200 mM NaCl, and 5 mM 2-mercaptoethanol. Glutathione Sepharose 4B beads (5 µL) were added to the reaction mixtures, which were gently mixed at 4°C for 60 min. The beads were then washed three times with 1 mL of wash buffer, containing 20 mM Tris-HCl (pH 8.0), 0.4 M NaCl, 10% glycerol, 5 mM 2-mercaptoethanol, and 0.05% Triton X-100. The proteins bound to the beads were separated by 7% SDS-PAGE, and were visualized by Coomassie Brilliant Blue staining.

In vitro FANCD2 monoubiquitination and purification of monoubiquitinated FANCD2

Experimental procedures are described in Supplementary Materials and Methods.

Gel-shift assay

Circular ϕ X174 dsDNA (100 ng) was mixed with cFANCD2 (0.45-1.8 μ M) or cFANCD2(1-1389) (0.45-1.8 μ M) in 10 μ L of reaction buffer, containing 22 mM Tris-HCl (pH 8.0), 70 mM NaCl, 7% glycerol, 2 mM $MgCl_2$, and 2.5 mM dithiothreitol. The samples were incubated at 37°C for 15 min, and were then analyzed by 0.8% agarose gel electrophoresis in TAE buffer. DNA was visualized by ethidium bromide staining.

siRNA transfection, immunoblotting, and fluorescence recovery after photobleaching (FRAP)

The FANCD2-specific Stealth RNAs (#1, HSS103525, AAUGAACGCUCUUUAGCAGACAUGG, for Figures 3 and 6, and Supplementary Figure S3; and #2, HSS103527, AAUAGACGACAACUUAUCCAUCACC, for Supplementary Figure S3; Invitrogen), a FANCA-specific Stealth RNA (AAGGGUCAAGAGGGAAAAUA, for Supplementary Figure S4; Invitrogen), and the control RNA (Negative Control Medium; Invitrogen) were introduced into HeLa cells expressing histone H3-GFP by LipofectAMINE2000 (Invitrogen), as described (Kimura *et al*, 2006). Total cellular proteins were prepared 0-4 days after the RNA introduction, separated by 8% SDS-PAGE, and immunoblotted with a mouse monoclonal antibody directed against either FANCD2 (1:250; FI17, Santa Cruz Biotechnology, Inc.), α -tubulin (1:1000; Sigma) as a loading control, or an antibody against FANCA (1:1000; rabbit polyclonal, Bethyl Laboratories, Inc.). Secondary detection was performed with a sheep anti-mouse IgG, horseradish peroxidase linked species-specific F(ab')₂ fragment (GE Healthcare; 1:500 for FANCD2, 1:1000 for α -tubulin) or a sheep anti-rabbit IgG,

horseradish peroxidase linked species-specific F(ab')₂ fragment (GE Healthcare; 1:1000 for FANCA). The signals were detected by chemiluminescence (Western Lightning Plus; Perkin Elmer).

To generate HeLa cells expressing both H3-GFP and mCherry-PCNA, cells expressing H3-GFP (blasticidin resistance; Kimura and Cook, 2001) and mCherry-PCNA (puromycin-resistance; generated using pMX-puro-based expression vector carrying mCherry-PCNA; Leonhardt *et al*, 2000; Kitamura *et al*, 2003) were fused using polyethylene glycol (Roche; Kimura and Cook, 2001) and single colonies were selected in the presence of 1 µg/ml blasticidin and 0.5 µg/ml puromycin.

FRAP was performed 3-4 days after siRNA transfection as described (Kimura *et al*, 2006), using a confocal microscope (FV-1000; Olympus) with a 60x UPlanSApo NA = 1.35 lens. Three confocal images of a field containing 4-10 nuclei were collected (800 × 800 pixels, zoom 3, scan speed 2 µs/pixel, pinhole 800 µm, Kalman filtration for 4 scans, LP505 emission filter, and 0.1% transmission of 488-nm Ar laser), one half of each nucleus was bleached using 75% transmission of 488 nm and 100% of 514 nm (2 iterations), and images were obtained using the original setting at 1 min intervals.

For the complementation experiments, RNAi-resistant *hFANCD2* genes were constructed by introducing silent mutations in the FANCD2-specific Stealth RNA (#1) target sequence (5'-TCT GCT AAA GAG-3'), as follows: 5'-TCC GCC AAG GAA-3'. HeLa cells expressing histone H3-GFP were transfected with the FANCD2-specific Stealth RNAs (#1) to knockdown the endogenous hFANCD2. The next day, the cells were transfected with the RNAi-resistant mCherry-*hFANCD2*,

mCherry-*hFANCD2(R302W)*, or mCherry-*hFANCD2(K561R)* gene, using the FuGENE system (Promega), and were further grown for 2 days before FRAP. After FRAP, the glass-bottom dish was processed for immunofluorescence.

The fluorescence intensity of the bleached area was measured using Image J 1.39u (W. Rasband; <http://rsb.info.nih.gov/ij/>). After subtracting the background, the intensity was normalized to the initial intensity before bleaching.

Immunofluorescence

For immunofluorescence, HeLa cells expressing histone H3-GFP were grown on a glass-bottom dish (Mat-tek), transfected with Stealth RNA, and fixed with 4% paraformaldehyde at 3 days after transfection. The fixed cells were permeabilized and stained using mouse anti-FANCD2 (1:250) and goat Cy3-conjugated anti-mouse IgG (1:500; Jackson ImmunoResearch). When mCherry-*hFANCD2* was transfected, goat Cy5-conjugated anti-mouse IgG (1:500; Jackson ImmunoResearch) was used as the secondary antibody. For double staining with FANCA, rabbit anti-FANCA (1:100; Bethyl Laboratories, Inc.) and goat Cy5-conjugated anti-rabbit IgG (1:500; Jackson ImmunoResearch) are also used. DNA was counterstained with DAPI. The fluorescence images were obtained using a confocal microscope (Olympus FV-1000 with a 60x UPlanSApo NA = 1.35 oil-immersion objective lens; or Carl Zeiss LSM510 with a 40x C-Apo NA = 1.2 water-immersion objective lens).

Generation of the FANCD2^{-/-} DT40 cells producing the FANCD2 mutants

Plasmids for the cFANCD2 mutant fusions were constructed in the pcDNA3.1-based GFP or histone H2B-GFP expression vector, by inserting FANCD2 fragments using the Gateway system (Invitrogen). To obtain stably expressing clones, these plasmids were transfected into *FANCD2*-deficient DT40 cells (Yamamoto *et al*, 2005), and the clones expressing the fusions were identified by measuring the GFP fluorescence with a FACSCalibur (Becton Dickinson), as described (Ishiai *et al*, 2004; Yamamoto *et al*, 2005).

Cisplatin sensitivity assay

Sensitivity to cisplatin (Nihon-Kayaku) was assayed by colony formation, in medium containing 1.4% methylcellulose and the indicated dosage of cisplatin (Ishiai *et al*, 2004; Yamamoto *et al*, 2005).

Cell fractionation and detection of FANCD2/FANCI proteins

The indicated cells were either treated with MMC (500 ng/ml for 6 hr) or left untreated, and were fractionated into soluble and chromatin fractions, as described previously (Matsushita *et al*, 2005; Ishiai *et al*, 2008). Pull-down assays of cFANCD2-H2B-GFP proteins were performed using anti-GFP beads (MBL). The proteins were separated by 6% SDS-PAGE, and were detected by western blotting. The anti-chicken FANCD2 and FANCI antibodies were obtained by immunizing rabbits with the bacterially expressed His fusion protein with full-length chicken FANCD2 and the GST fusion protein with chicken FANCI (amino acids 1-251), respectively.

Gel filtration analysis

The purified cFANCD2 and cFANCI proteins were analyzed by Superdex 200 HR 10/30 (GE Healthcare) gel filtration chromatography. The elution buffer contained 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 10% glycerol and 1 mM DTT.

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Author contributions

K.S., K.T., S.F., Y.T., A.O., H.T., and W.K. purified FANCD2, FANCI, and histones, and K.S. and K.T. performed biochemical analyses. M.I., H.Kitao, and M.T. performed genetic and cell biological analyses. K.S. and H.Kimura performed FRAP analyses. K.S.

and N.D. performed domain analysis of FANCD2 by mass spectroscopy. A.O., H.T., and C.O. performed proteome analysis of histone binding proteins. H. Kurumizaka and M.T. conceived, designed, and supervised all of the work, and K.S. and H. Kurumizaka wrote the paper. All of the authors discussed the results and commented on the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Ali AM, Pradhan A, Singh TR, Du C, Li J, Wahengbam K, Grassman E, Auerbach AD, Pang Q, Meetei AR (2012) FAAP20: a novel ubiquitin-binding FA nuclear core-complex protein required for functional integrity of the FA-BRCA DNA repair pathway. *Blood* **119**: 3285-3294
- Avvakumov N, Nourani A, Côté J (2011) Histone Chaperones: Modulators of Chromatin Marks. *Mol Cell* **41**: 502-514
- Bhagwat N, Olsen AL, Wang AT, Hanada K, Stuckert P, Kanaar R, D'Andrea A, Niedernhofer LJ, McHugh PJ (2009) XPF-ERCC1 participates in the Fanconi anemia pathway of cross-link repair. *Mol Cell Biol* **29**: 6427-6437
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254

Crossan GP, van der Weyden L, Rosado IV, Langevin F, Gaillard PH, McIntyre RE; Sanger Mouse Genetics Project, Gallagher F, Kettunen MI, Lewis DY, Brindle K, Arends MJ, Adams DJ, Patel KJ (2011) Disruption of mouse Slx4, a regulator of structure-specific nucleases, phenocopies Fanconi anemia. *Nat Genet* **43**: 147–152

Dunleavy EM, Roche D., Tagami H, Lacoste N, Ray-Gallet D, Nakamura Y, Daigo Y, Nakatani Y, Almouzni G (2009). HJURP is a cell-cycle-dependent maintenance and deposition factor of CENP-A at centromeres. *Cell* **137**: 485-497

English CM, Adkins MW, Carson JJ, Churchill ME, Tyler JK. (2006) Structural basis for the histone chaperone activity of Asf1. *Cell* **127**: 495-508

Fekairi S, Scaglione S, Chahwan C, Taylor ER, Tissier A, Coulon S, Dong MQ, Ruse C, Yates JR 3rd, Russell P, Fuchs RP, McGowan CH, Gaillard PH (2009) Human SLX4 is a Holliday junction resolvase subunit that binds multiple DNA repair/recombination endonucleases. *Cell* **138**: 78-89

Foltz DR, Jansen LET, Bailey AO, Yates JR, Bassett EA, Wood S, Black BE, Cleveland DW (2009) Centromere-specific assembly of CENP-A nucleosomes is mediated by HJURP. *Cell* **137**: 472-484

Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn MS, Timmers C, Hejna J, Grompe M, D'Andrea AD (2001) Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol Cell* **7**: 249-262

Garner E, Smogorzewska A (2011) Ubiquitylation and the Fanconi anemia pathway. *FEBS Lett* **585**: 2853-2860

Hejna J, Holtorf M, Hines J, Mathewson L, Hemphill A, Al-Dhalimy M, Olson SB, Moses RE (2008) Tip60 is required for DNA interstrand cross-link repair in the Fanconi anemia pathway. *J Biol Chem* **283**: 9844-9851

Hicks JK, Chute CL, Paulsen MT, Ragland RL, Howlett NG, Guéranger Q, Glover TW, Canman CE (2010) Differential roles for DNA polymerases eta, zeta, and REV1 in lesion bypass of intrastrand versus interstrand DNA cross-links. *Mol Cell Biol* **30**: 1217-1230

Hu H, Liu Y, Wang M, Fang J, Huang H, Yang N, Li Y, Wang J, Yao X, Shi Y, Li G, Xu RM (2011) Structure of a CENP-A-histone H4 heterodimer in complex with chaperone HJURP. *Genes Dev* **25**: 901-906

Ishiai M, Kimura M, Namikoshi K, Yamazoe M, Yamamoto K, Arakawa H, Agematsu K, Matsushita N, Takeda S, Buerstedde JM, Takata M (2004) DNA cross-link repair protein SNM1A interacts with PIAS1 in nuclear focus formation. *Mol Cell Biol* **24**: 10733-10741

Ishiai M, Kitao H, Smogorzewska A, Tomida J, Kinomura A, Uchida E, Saberi A, Kinoshita E, Kinoshita-Kikuta E, Koike T, Tashiro S, Elledge SJ, Takata M (2008) FANCI phosphorylation functions as a molecular switch to turn on the Fanconi anemia pathway. *Nat Struct Mol Biol* **15**: 1138-1146

Kee Y, D'Andrea AD (2010) Expanded roles of the Fanconi anemia pathway in preserving genomic stability. *Genes Dev* **24**: 1680-16943

Kim H, Yang K, Dejsuphong D, D'Andrea AD (2012) Regulation of Rev1 by the Fanconi anemia core complex. *Nat Struct Mol Biol* **19**: 164-170

Kim Y, Lach FP, Desetty R, Hanenberg H, Auerbach AD, Smogorzewska A (2011) Mutations of the SLX4 gene in Fanconi anemia. *Nat Genet* **43**: 142-146

Kimura H, Cook PR (2001) Kinetics of core histones in living human cells: little exchange of H3 and H4 and some rapid exchange of H2B. *J Cell Biol* **153**: 1341-1353

Kimura H, Takizawa N, Allemand E, Hori T, Iborra FJ, Nozaki N, Muraki M, Hagiwara M, Krainer AR, Fukagawa T, Okawa K (2006) A novel histone-exchange factor, protein phosphatase 2Cγ, mediates the exchange and dephosphorylation of H2A-H2B. *J Cell Biol* **175**: 389-400

Kitamura T, Koshino Y, Shibata F, Oki T, Nakajima H, Nosaka T, Kumagai H (2003) Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. *Exp Hematol* **31**: 1007-1014.

Kitao H, Takata M (2011) Fanconi anemia: a disorder defective in the DNA damage response. *Int J Hematol* **93**: 417-424

Knipscheer P, Räschle M, Smogorzewska A, Enoiu M, Ho TV, Schärer OD, Elledge SJ, Walter JC (2009) The Fanconi anemia pathway promotes replication-dependent DNA interstrand cross-link repair. *Science* **326**: 1698-1701

Kratz K, Schöpf B, Kaden S, Sandoel A, Eberhard R, Lademann C, Cannavó E, Sartori AA, Hengartner MO, Jiricny J (2010) Deficiency of FANCD2-associated nuclease KIAA1018/FAN1 sensitizes cells to interstrand crosslinking agents. *Cell* **142**: 77-88

Leonhardt H, Rahn HP, Weinzierl P, Sporbert A, Cremer T, Zink D, Cardoso MC (2000) Dynamics of DNA replication factories in living cells. *J Cell Biol* **149**: 271-280

Leung JW, Wang Y, Fong KW, Huen MS, Li L, Chen J. (2012) Fanconi anemia (FA) binding protein FAAP20 stabilizes FA complementation group A (FANCA) and participates in interstrand cross-link repair. *Proc Natl Acad Sci U S A* **109**: 4491-4496

MacKay C, Déclais AC, Lundin C, Agostinho A, Deans AJ, MacArtney TJ, Hofmann K, Gartner A, West SC, Helleday T, Lilley DM, Rouse J (2010) Identification of KIAA1018/FAN1, a DNA repair nuclease recruited to DNA damage by monoubiquitinated FANCD2. *Cell* **142**: 65-76

Matsushita N, Kitao H, Ishiai M, Nagashima N, Hirano S, Okawa K, Ohta T, Yu DS, McHugh PJ, Hickson ID, Venkitaraman AR, Kurumizaka H, Takata M (2005) A FancD2-monoubiquitin fusion reveals hidden functions of Fanconi anemia core complex in DNA repair. *Mol Cell* **19**: 841-847

Montes de Oca R, Andreassen PR, Margossian SP, Gregory RC, Taniguchi T, Wang X, Houghtaling S, Grompe M, D'Andrea AD (2005) Regulated interaction of the Fanconi anemia protein, FANCD2, with chromatin. *Blood* **105**: 1003-1009

Natsume R, Eitoku M, Akai Y, Sano N, Horikoshi M, Senda T. (2007) Structure and function of the histone chaperone CIA/ASF1 complexed with histones H3 and H4. *Nature* **446**: 338-341

Niedernhofer LJ, Lalai AS, Hoeijmakers JHJ (2005) Fanconi anemia (cross)linked to DNA repair. *Cell* **123**: 1191-1198

Nomura Y, Adachi N, Koyama H (2007) Human Mus81 and FANCB independently contribute to repair of DNA damage during replication. *Genes Cells* **12**: 1111-1122

Nozawa RS, Nagao K, Masuda HT, Iwasaki O, Hirota T, Nozaki N, Kimura H, Obuse C. (2010) Human POGZ modulates dissociation of HP1alpha from mitotic chromosome arms through Aurora B activation. *Nat Cell Biol* **12**: 719-727

Osakabe A, Tachiwana H, Matsunaga T, Shiga T, Nozawa RS, Obuse C, Kurumizaka H (2010) Nucleosome formation activity of human somatic nuclear autoantigenic sperm protein (sNASP). *J Biol Chem* **285**: 11913-11921

Pace P, Mosedale G, Hodskinson MR, Rosado IV, Sivasubramaniam M, Patel KJ (2010) Ku70 corrupts DNA repair in the absence of the Fanconi anemia pathway. *Science* **329**: 219-223

Sato K, Toda K, Ishiai M, Takata M, Kurumizaka H (2012) DNA robustly stimulates FANCD2 monoubiquitylation in the complex with FANCI. *Nucleic Acids Res* **40**: 4553-4561

Seki S, Ohzeki M, Uchida A, Hirano S, Matsushita N, Kitao H, Oda T, Yamashita T, Kashiwara N, Tsubahara A, Takata M, Ishiai M (2007) A requirement of FancL and FancD2 monoubiquitination in DNA repair. *Genes Cells* **12**: 299-310

Sims AE, Spiteri E, Sims RJ 3rd, Arita AG, Lach FP, Landers T, Wurm M, Freund M, Neveling K, Hanenberg H, Auerbach AD, Huang TT (2007) FANCI is a second monoubiquitinated member of the Fanconi anemia pathway. *Nat Struct Mol Biol* **14**: 564-567

Smogorzewska A, Matsuoka S, Vinciguerra P, McDonald ER 3rd, Hurov KE, Luo J, Ballif BA, Gygi SP, Hofmann K, D'Andrea AD, Elledge SJ (2007) Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. *Cell* **129**: 289-301

Smogorzewska A, Desetty R, Saito TT, Schlabach M, Lach FP, Sowa ME, Clark AB, Kunkel TA, Harper JW, Colaiácovo MP, Elledge SJ (2010) A genetic screen identifies FAN1, a Fanconi anemia-associated nuclease necessary for DNA interstrand crosslink repair. *Mol Cell* **39**: 36-47

Stoepker C, Hain K, Schuster B, Hilhorst-Hofstee Y, Rooimans MA, Steltenpool J, Oostra AB, Eirich K, Korthof ET, Nieuwint AW, Jaspers NG, Bettecken T, Joenje H, Schindler D, Rouse J, de Winter JP (2011) SLX4, a coordinator of structure-specific endonucleases, is mutated in a new Fanconi anemia subtype. *Nat Genet* **43**: 138-141

Svendsen JM, Smogorzewska A, Sowa ME, O'Connell BC, Gygi SP, Elledge SJ, Harper JW (2009) Mammalian BTBD12/SLX4 assembles a Holliday junction resolvase and is required for DNA repair. *Cell* **138**: 63-77

Tanaka Y, Tawaramoto-Sasanuma M, Kawaguchi S, Ohta T, Yoda K, Kurumizaka H, Yokoyama S (2004) Expression and purification of recombinant human histones. *Methods (San Diego, Calif)* **33**: 3-11

Tachiwana H, Osakabe A, Kimura H, Kurumizaka H. (2008) Nucleosome formation with the testis-specific histone H3 variant, H3t, by human nucleosome assembly proteins in vitro. *Nucleic Acids Res.* **36**: 2208-2218

Tachiwana H, Kagawa W, Osakabe A, Kawaguchi K, Shiga T, Hayashi-Takanaka Y, Kimura H, Kurumizaka H (2010) Structural basis of instability of the nucleosome containing a testis-specific histone variant, human H3T. *Proc Natl Acad Sci USA* **107**: 10454-10459

Tachiwana H, Kagawa W, Shiga T, Osakabe A, Miya Y, Saito K, Hayashi-Takanaka Y, Oda T, Sato M, Park S-Y, Kimura H, Kurumizaka H (2011a). Crystal structure of the human centromeric nucleosome containing CENP-A. *Nature* **476**: 232-235

Tachiwana H, Osakabe A, Shiga T, Miya Y, Kimura H, Kagawa W, Kurumizaka H (2011b) Structures of human nucleosomes containing major histone H3 variants. *Acta Cryst D* **67(Pt 6)**: 578-583

Taniguchi T, D'Andrea AD (2006) Molecular pathogenesis of Fanconi anemia: recent progress. *Blood* **107**: 4223-4233

Thompson LH, Hinz JM, Yamada NA, Jones NJ (2005) How Fanconi anemia proteins promote the four Rs: replication, recombination, repair, and recovery. *Environ Mol Mutagen* **45**: 128-142

Timmers C, Taniguchi T, Hejna J, Reifsteck C, Lucas L, Bruun D, Thayer M, Cox B, Olson S, D'Andrea AD, Moses R, Grompe M (2001) Positional cloning of a novel Fanconi anemia gene, FANCD2. *Mol Cell* **7**: 241-248.

Vaz F, Hanenberg H, Schuster B, Barker K, Wiek C, Erven V, Neveling K, Endt D, Kesterton I, Autore F, Fraternali F, Freund M, Hartmann L, Grimwade D, Roberts RG, Schaal H, Mohammed S, Rahman N, Schindler D, Mathew CG (2010) Mutation of the RAD51C gene in a Fanconi anemia-like disorder. *Nat Genet* **42**: 406-409

Venkitaraman AR (2004) Tracing the network connecting BRCA and Fanconi anaemia proteins. *Nat Rev Cancer* **4**: 435-445

Wang W (2007) Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. *Nat Rev Genet* **8**: 735-748

Wolffe A (1998) Chromatin: Structure and Function. Third Edition. Academic Press, San Diego, California, USA

Yamamoto K, Ishiai M, Matsushita N, Arakawa H, Lamerdin JE, Buerstedde JM, Tanimoto M, Harada M, Thompson LH, Takata M (2003) Fanconi anemia FANCG protein in mitigating radiation- and enzyme-induced DNA double-strand breaks by homologous recombination in vertebrate cells. *Mol Cell Biol* **23**: 5421-5430

Yamamoto K, Hirano S, Ishiai M, Morishima K, Kitao H, Namikoshi K, Kimura M, Matsushita N, Arakawa H, Buerstedde JM, Komatsu K, Thompson LH, Takata M (2005) Fanconi anemia protein FANCD2 promotes immunoglobulin gene conversion and DNA repair through a mechanism related to homologous recombination. *Mol Cell Biol* **25**: 34-43

Yamamoto KN, Kobayashi S, Tsuda M, Kurumizaka H, Takata M, Kono K, Jiricny J, Takeda S, Hirota K (2011) Involvement of SLX4 in interstrand cross-link repair is regulated by the Fanconi anemia pathway. *Proc Natl Acad Sci USA* **108**: 6492-6496

Yoshikiyo K, Kratz K, Hirota K, Nishihara K, Takata M, Kurumizaka H, Horimoto S, Takeda S, Jiricny J (2010) KIAA1018/FAN1 nuclease protects cells against genomic instability induced by interstrand cross-linking agents. *Proc Natl Acad Sci USA* **107**: 21553-21557

Figure Legends

Figure 1 hFANCD2 promotes nucleosome assembly. **(A)** The H3/H4-conjugated beads were incubated with a HeLa whole cell extract (WCE), and the endogenous FANCD2 bound to the beads was detected by western blotting with an anti-FANCD2 monoclonal antibody (α -FANCD2). N and H indicate the control Affi-Gel 10 beads and the H3/H4-conjugated beads, respectively. Input WCE (30 μ g of protein) and hFANCD2 (125 ng) were applied in lanes 4 and 5, respectively. **(B)** The H3/H4 beads were incubated with purified hFANCD2 in the absence or presence of DNaseI, washed with buffer, and mixed with 2-fold SDS sample buffer. The proteins bound to the beads were analyzed by 15% SDS-PAGE. **(C)** Topological assay. A schematic diagram of topological assay is shown in the left panel. Nucleosomes were reconstituted on the relaxed plasmid DNA by hFANCD2 (0.2, 0.5, and 0.9 μ M), in the presence of wheat germ topoisomerase I. After deproteinization, the topoisomers were separated by agarose gel electrophoresis. Highly supercoiled and relaxed DNAs are denoted as “sc” and “relaxed”, respectively. **(D)** Nucleosome assembly assay. A schematic diagram of the nucleosome assembly assay is shown in the left panel. Nucleosomes were reconstituted on the linear 195 base-pair DNA by hNap1 (0.4, 0.8, and 1.6 μ M) or hFANCD2 (0.2, 0.4, and 0.8 μ M). Nucleosomes positioned at the edge and center of the 195 bp DNA are indicated by cartoons on the right side of the panel.

Figure 2 The C-terminal region of FANCD2 is responsible for histone binding and nucleosome assembly. **(A)** Schematic representations of full-length cFANCD2, and the

cFANCD2(1-1389), cFANCD2(1-1267), cFANCD2(1-1167), cFANCD2(669-1439), and cFANCD2(953-1439) deletion mutants. The cFANCD2 domains, solenoid 1, helical domain 1, solenoid 2, helical domain 2, solenoid 3, and solenoid 4, are denoted as S1, HD1, S2, HD2, S3, and S4, respectively (Joo *et al.* 2011). The FANCD2 C-terminal acidic region is colored red. The amino acid sequences of the C-terminal regions of the *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Xenopus laevis*, *Danio rerio*, and *Drosophila melanogaster* FANCD2 proteins are aligned. The highly conserved residues are colored red. The mutated residues in cFANCD2(R1336A/K1346A) and cFANCD2(D1350A/E1365A/E1382A) are indicated by orange and purple arrowheads, respectively. **(B)** The H3/H4 beads were incubated with extracts of HEK293T cells, producing either GFP-tagged cFANCD2, cFANCD2(1-1389), cFANCD2(1-1267), cFANCD2(1-1167), cFANCD2(669-1439), cFANCD2(953-1439), cFANCD2(R305W), cFANCD2(K563R), cFANCD2(D1350A/E1365A/E1382A), or cFANCD2(R1336A/K1346A). Proteins bound to the beads were detected by western blotting with an anti-chFANCD2 (polyclonal) antibody. The bottom panel indicates negative control experiments with beads lacking histones. **(C)** Nucleosomes were reconstituted on the relaxed plasmid DNA by cFANCD2 (lanes 3-7), cFANCD2(1-1389) (lanes 8-12), cFANCD2(1-1267) (lanes 13-17), cFANCD2(1-1167) (lanes 18-22), and cFANCD2(953-1439) (lanes 23-27) in the presence of wheat germ topoisomerase I. After deproteinization, the topoisomers were separated by agarose gel electrophoresis with ethidium bromide staining. The cFANCD2 concentrations were 0, 0.45, 0.90, and 1.8 μ M. Highly supercoiled and relaxed DNAs are denoted as “sc” and “relaxed”, respectively.

(D) Graphic representation of nucleosome assembly activities of the cFANCD2 mutants shown in panel C. Representative images are shown in panel C. The supercoiled DNA fractions were generated by nucleosome assembly in the presence of cFANCD2, and the intensities of the bands indicated by the arrows in panel C were quantitated by an LAS-4000 Image Analyzer (GE Healthcare). Means of three independent experiments are shown with standard deviations. (E) Nucleosomes were reconstituted on the relaxed plasmid DNA by cFANCD2 (lanes 3-7), cFANCD2(R1336A/K1346A) (lanes 8-12), and cFANCD2(D1350A/E1365A/E1382A) (lanes 13-17) in the presence of wheat germ topoisomerase I. The cFANCD2 concentrations were 0, 0.45, 0.90, and 1.8 μ M. Highly supercoiled and relaxed DNAs are denoted as “sc” and “relaxed”, respectively. (F) Graphic representation of the nucleosome assembly activities of the cFANCD2 mutants shown in panel E. Representative images are shown in panel E. The supercoiled DNA fractions were generated by nucleosome assembly in the presence of cFANCD2, and the intensities of the bands indicated by the arrows in panel E were quantitated by an LAS-4000 Image Analyzer (GE Healthcare). Means of three independent experiments are shown with standard deviations.

Figure 3 Histone H3 mobility is decreased in FANCD2-knockdown cells in the presence of a DNA crosslinking reagent. (A, B) FRAP with HeLa cells. Three days after the transfection of hFANCD2-siRNA or control RNA, the mobility of histone H3-GFP was analyzed by bleaching one-half of a nucleus in the absence (A) or presence (B) of 50 ng/mL MMC for 12-18 h. The mean of the relative fluorescence intensity with the

standard deviation ($n = 10-11$) and examples are shown. (C) FRAP with HeLa cells stably expressing mCherry-PCNA. Three days after the transfection of control RNA, the mobility of histone H3-GFP was analyzed in the absence of MMC. The PCNA foci-positive (S-phase) cells were identified by the characteristic mCherry-PCNA distribution. The mean of the relative fluorescence intensity with the standard deviation ($n = 10-14$) and examples are shown. (D) FRAP with MMC-treated HeLa cells expressing H3-GFP and mCherry-PCNA in S-phase. Three days after the transfection of hFANCD2-siRNA or control RNA, the mobility of histone H3-GFP in the PCNA foci-positive (S-phase) cells was analyzed in the presence of 50 ng/mL MMC. The mean of the relative fluorescence intensity with the standard deviation ($n = 10-18$) and examples are shown. Bars: 10 μm .

Figure 4 DNA repair defects in the cFANCD2 C-terminal mutants. (A) Colony survival assay of the cFANCD2^{-/-} DT40 cells expressing GFP fusions with the wild type (WT) and indicated cFANCD2 mutants in the presence of cisplatin. The mean and standard deviation of measurements performed in triplicate are shown. (B) FANCD2/FANCI monoubiquitination in cFANCD2^{-/-} cells expressing the indicated cFANCD2 mutants. Cells were treated with or without MMC, and the whole cell extracts were subjected to western blotting. The bands detected just above the original bands (S-forms) correspond to the monoubiquitinated forms (L-forms) of cFANCD2 and cFANCI (lanes 2, 6, 8 and 14). The L-form and S-form bands were quantitated with the Image J software, and the L/S ratios are indicated just above each panel. An asterisk indicates the non-specific

bands. ND: not detectable. (C) Colony survival assay of the cFANCD2^{-/-} cells expressing H2B-GFP fusions with the WT and indicated cFANCD2 mutants in the presence of cisplatin. The mean and standard deviation of measurements performed in triplicate are shown. (D) Chromatin targeting of cFANCD2 (WT)-H2B, cFANCD2(1-1167)-H2B, cFANCD2(1-1267)-H2B, and cFANCD2(R1336A/K1346A)-H2B. Negative control experiments in the absence of exogenously expressed cFANCD2 are shown in the top panel. The cFANCD2^{-/-} cells expressing the indicated proteins were treated with MMC (500 ng/ml, 6 hr) or left untreated and then fractionated. Each fraction was separated by SDS-PAGE, and western blotting was performed using anti-cFANCD2 and anti-cFANCI antibodies. An asterisk indicates the non-specific band.

Figure 5 An FA-related mutant, cFANCD2(R305W), is defective in nucleosome assembly activity and DNA repair. (A) Nucleosomes were reconstituted on the relaxed plasmid DNA by cFANCD2 (lanes 3-7) and cFANCD2(R305W) (lanes 8-12), in the presence of wheat germ topoisomerase I. After deproteinization, the topoisomers were separated by agarose gel electrophoresis. The cFANCD2 concentrations were 0, 0.45, 0.90, and 1.8 μ M. Highly supercoiled and relaxed DNAs are denoted as “sc” and “relaxed”, respectively. (B) Graphic representation of the nucleosome assembly activity of the cFANCD2(R305W) mutants. Representative images are shown in panel A. The supercoiled DNA fractions were generated by nucleosome assembly in the presence of cFANCD2 (indicated by arrows in panel A), and the intensities of the bands indicated by

the arrows in panel A were quantitated by an LAS-4000 Image Analyzer (GE Healthcare). Means of three independent experiments are shown with standard deviations. (C) FANCD2/FANCI monoubiquitination in cFANCD2^{-/-} cells expressing the wild type (WT) FANCD2 or cFANCD2(R305W). Cells were treated with or without MMC, and the whole cell extracts were subjected to western blotting. The bands detected just above the original bands (S-forms) correspond to the monoubiquitinated forms (L-forms) of cFANCD2 and cFANCI (lanes 2 and 4). The L-form and S-form bands were quantitated with the Image J software, and the L/S ratios are indicated just above each panel. An asterisk indicates the non-specific band. (D) Colony survival assay of the cFANCD DT40 cells expressing GFP fusions with the wild type (WT) and cFANCD2(R305W), in the presence of cisplatin. Four independent DT40 cells expressing cFANCD2(R305W) were tested and plotted. (E) Colony survival assay of the cFANCD^{-/-} DT40 cells expressing H2B-GFP fusions with the wild type (WT) and cFANCD2(R305W), in the presence of cisplatin. The mean and standard deviation of measurements performed in triplicate are shown.

Figure 6 An FA-related mutant, human FANCD2(R302W), is defective in facilitating histone exchange. (A-C) FRAP with HeLa cells expressing H3-GFP and mCherry fusions with hFANCD2 (WT) (A), hFANCD2(K561R) (B), and hFANCD2(R302W) (C). After the depletion of endogenous hFANCD2 by siRNA, mCherry-hFANCD2, hFANCD2(K561R), or -hFANCD2(R302W) was expressed. In these cells, the mobility of histone H3-GFP was analyzed in the presence of 50 ng/mL MMC for 12-18 h. The

mean of the relative fluorescence intensity with the standard deviation ($n = 10-12$) and examples are shown. Bars: 10 μm .

Figure 7 FANCI stimulates the FANCD2-mediated nucleosome assembly. **(A)** Gel filtration analysis of cFANCI-cFANCD2 complex formation. The SDS-PAGE analysis of the cFANCI-cFANCD2 fractions is shown below the gel filtration profiles. **(B, C)** Nucleosomes were reconstituted with relaxed plasmid DNA and cFANCD2 and/or cFANCI in the presence of wheat germ topoisomerase I. After deproteinization, the topoisomers were separated by agarose gel electrophoresis. The protein concentrations used in the assay are indicated at the top of the panels. Highly supercoiled and relaxed DNAs are denoted as “sc” and “relaxed”, respectively. **(D)** Graphic representation of the experiments shown in panel C, lanes 3-5. The supercoiled DNA fractions generated by nucleosome assembly in the presence of cFANCD2 and cFANCI (indicated by arrows in C) were quantitated. Means of three independent experiments are shown with standard deviations.

Figure 8 Models for the FANCD2 function in the FA pathway. **(A)** Locations of the Arg1334 and Lys1344 residues in the mouse FANCD2 structure. Structure of the mouse ID complex (PDB ID: 3S4W; Joo *et al*, 2011). The FANCD2 and FANCI subunits are colored gray and light blue, respectively. The FANCD2 Arg1334 and Lys1344 residues (red), corresponding to the cFANCD2 Arg1336 and Lys1346 residues, respectively, are located on the concave surface of the FANCD2 Solenoid 4 (S4) domain (dark gray). **(B)**

Three possible functions of the histone chaperone activity of the ID complex. (I) After the chromatin binding and monoubiquitination of the ID complex, the nucleosomes assembled by the ID complex on newly synthesized DNA may suppress the improper nucleolytic digestion by the repair nucleases, such as FAN1 and the SLX4 nuclease complex, which are recruited by the monoubiquitinated ID complex. (II) Chromatin remodeling mediated by the ID complex and FANCD2-associated chromatin modifiers may facilitate the RAD51-mediated homologous recombination reaction in chromatin. (III) The nucleosome assembly activity of the ID complex may be required for chromatin reconstitution after DNA repair. RAD51 and RPA are colored red and green, respectively. Histone octamers are shown in brown.

Figure 1

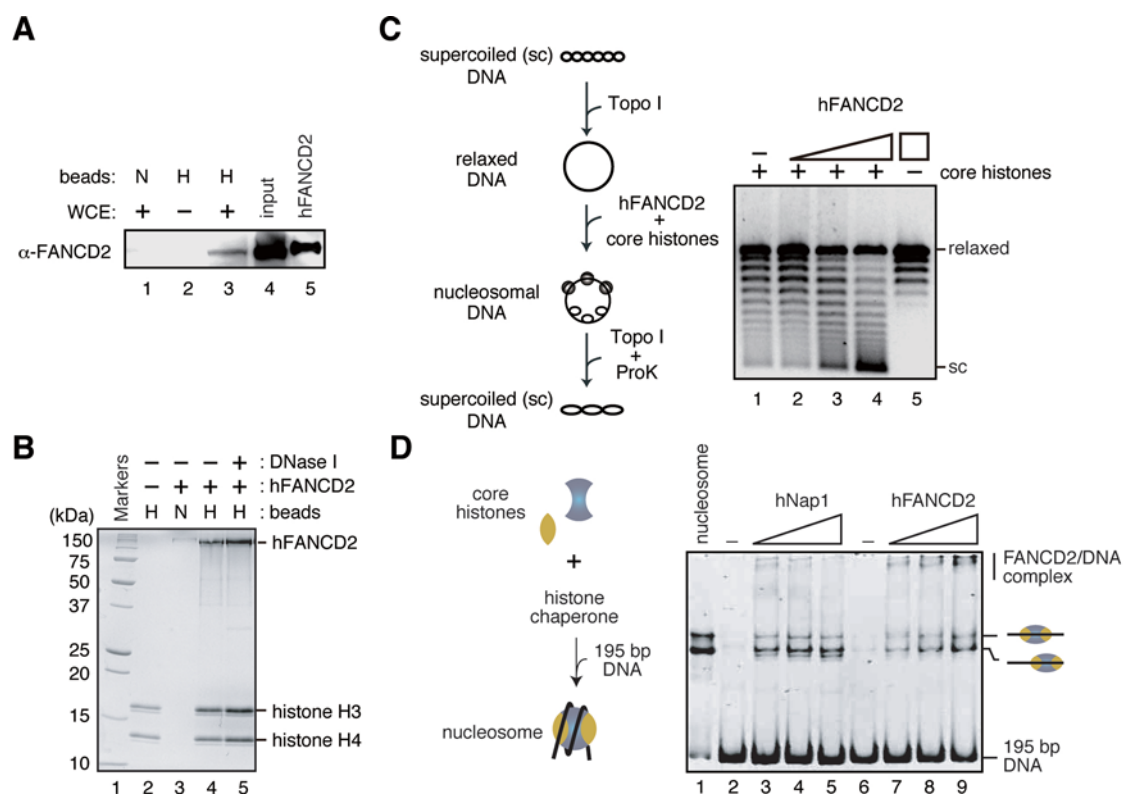


Figure 2

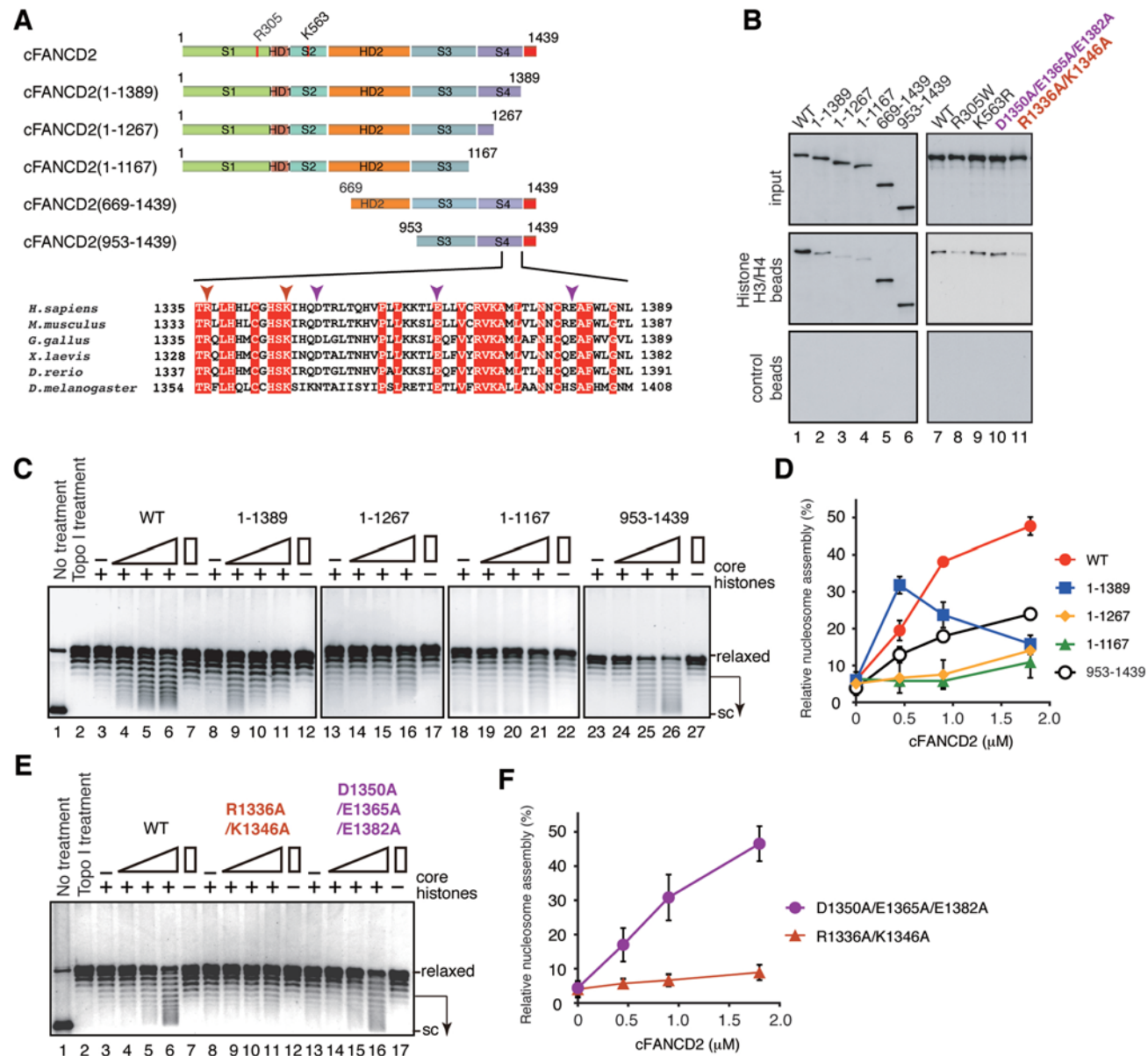


Figure 3

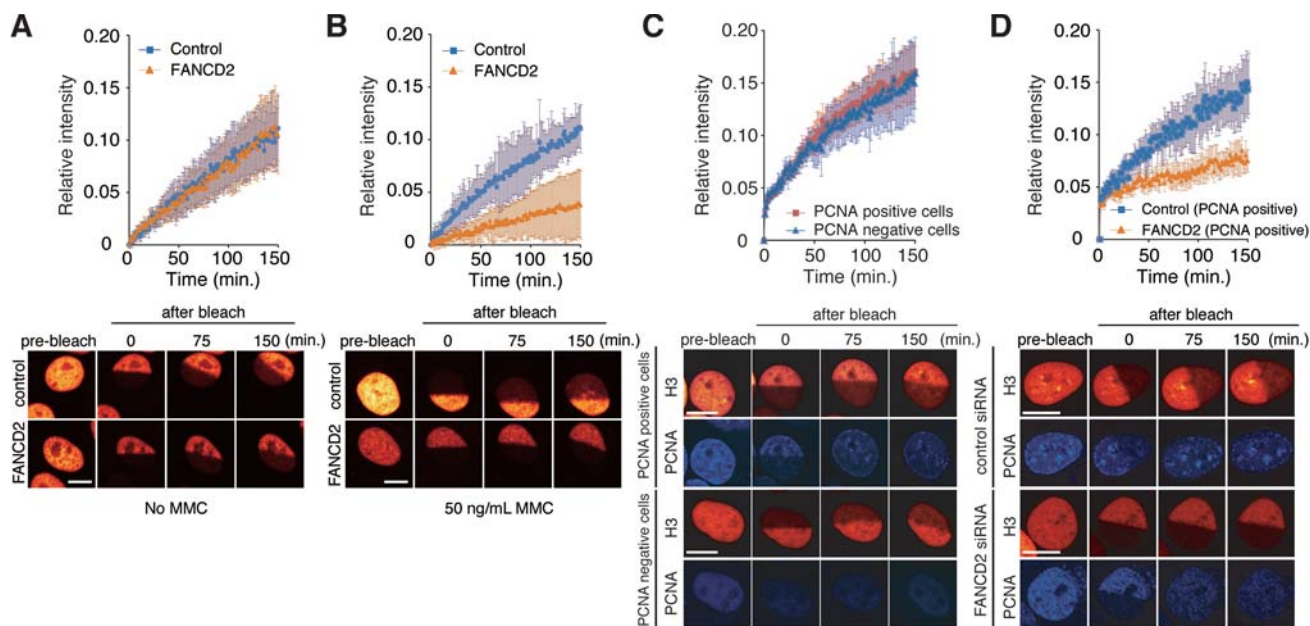


Figure 4

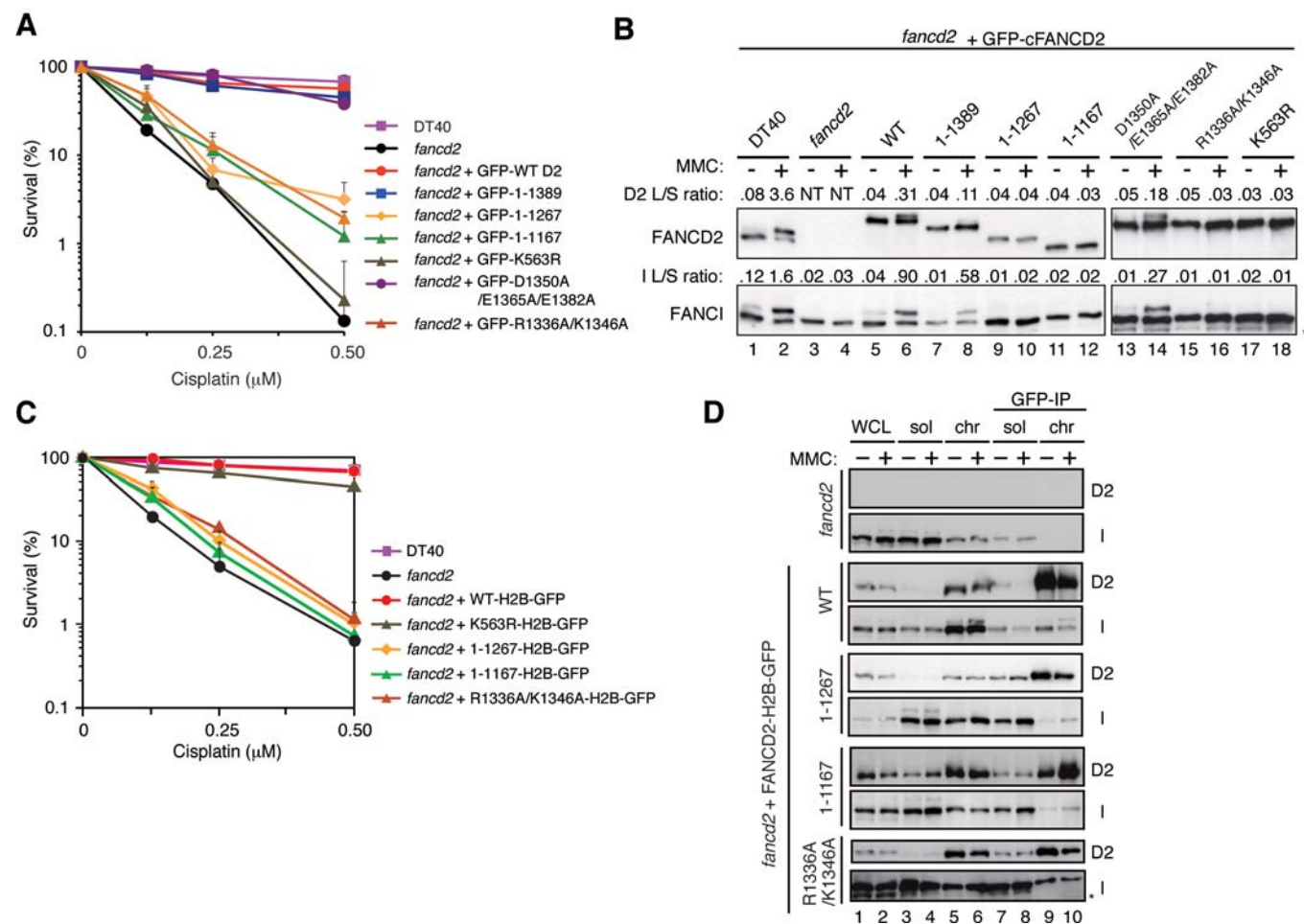


Figure 5

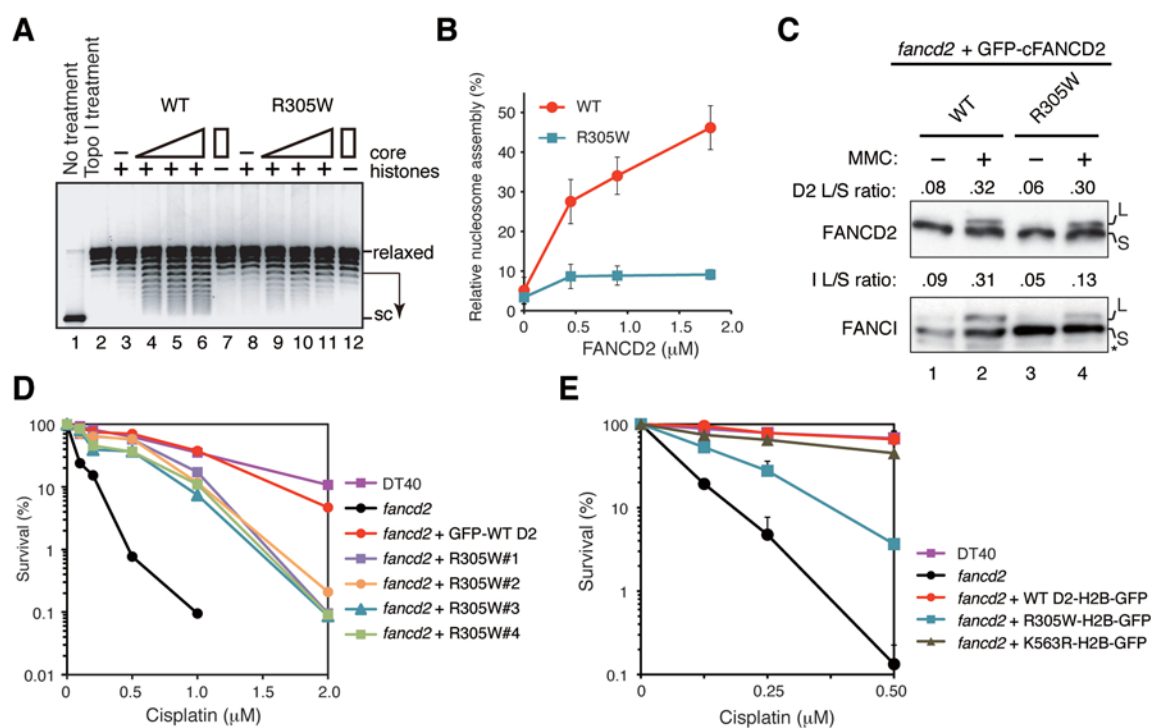


Figure 6

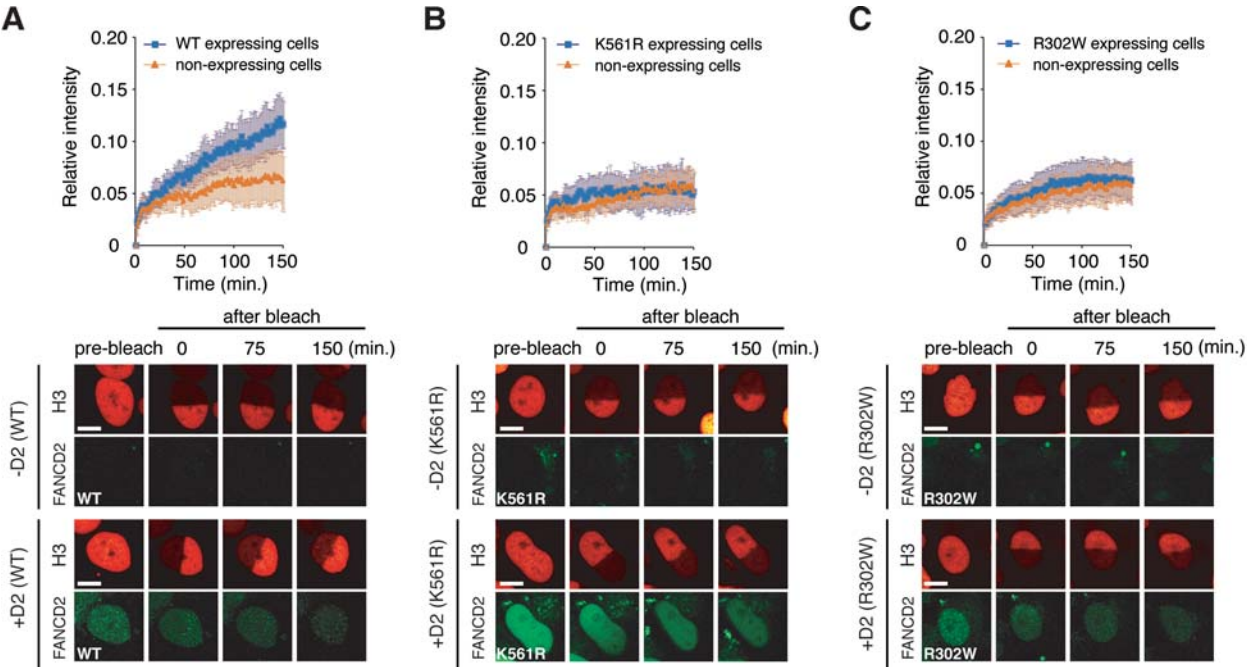


Figure 7

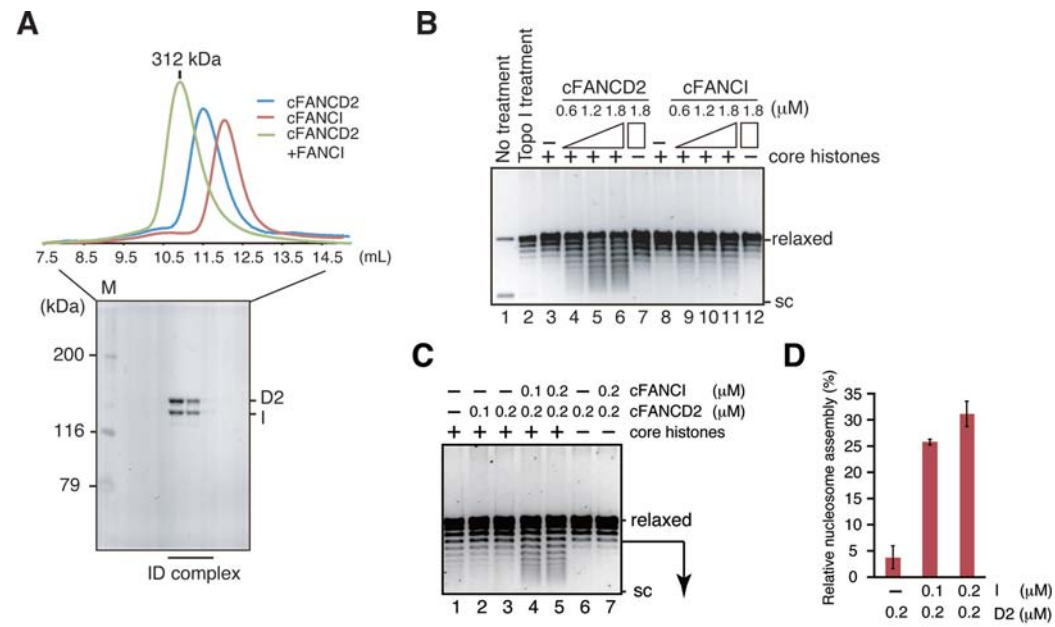


Figure 8

